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(54) Title: ANTISENSE MODULATION OF CASEIN KINASE 2-BETA EXPRESSION

(57) Abstract: Antisense compounds, compositions and methods are provided for modulating the expression of Casein kinase 2-beta. The compositions comprise antisense compounds, particularly antisense oligonucleotides, targeted to nucleic acids encoding Casein kinase 2-beta. Methods of using these compounds for modulation of Casein kinase 2-beta expression and for treatment of diseases associated with expression of Casein kinase 2-beta are provided.



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**ANTISENSE MODULATION OF CASEIN KINASE 2-BETA EXPRESSION  
FIELD OF THE INVENTION**

The present invention provides compositions and methods  
5 for modulating the expression of Casein kinase 2-beta. In  
particular, this invention relates to compounds,  
particularly oligonucleotides, specifically hybridizable  
with nucleic acids encoding Casein kinase 2-beta. Such  
compounds have been shown to modulate the expression of  
10 Casein kinase 2-beta.

**BACKGROUND OF THE INVENTION**

The process of phosphorylation, defined as the  
attachment of a phosphate moiety to a biological molecule  
through the action of enzymes called kinases, represents a  
15 major course by which intracellular signals are propagated  
resulting finally in a cellular response. Within the cell,  
these enzymes are generally classified into a protein-  
serine/threonine subfamily or a protein-tyrosine subfamily  
on the basis of phosphorylation substrate specificity. The  
20 extent of protein phosphorylation, in turn, is regulated by  
the opposing action of phosphatases which remove the  
phosphate moieties. Because phosphorylation is such a  
ubiquitous process within cells and because cellular  
phenotypes are largely influenced by the activity of these  
25 pathways, it is currently believed that a number of disease  
states and/or disorders are a result of either aberrant  
activation of, or functional mutations in, kinases.  
Consequently, considerable attention has been devoted to the  
study of kinases and their role in disease processes.

30 The protein kinases comprise an exceptionally large  
family of eukaryotic proteins which mediate the responses of  
cells to external stimuli and to date, in excess of several  
hundred unique members of the protein kinase family from a

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wide variety of eukaryotic organisms have been described and characterized at the amino acid sequence level.

Among the enzymes within the protein-serine/threonine kinase subfamily are two distinct casein kinases which have  
5 been designated casein kinase I (CKI) and casein kinase II (CKII or CK2) identified by the order of their elution from DEAE-cellulose. The casein kinases are distinguished from other protein kinases by their ability to phosphorylate serine or threonine residues within acidic recognition  
10 sequences such as found in casein and each has been thoroughly characterized regarding their physicochemical properties, recognition sequences, substrate specificity and effects on metabolic regulation. These enzymes have been found throughout the cell and their activities have been  
15 purified from or found to be associated with cytoplasmic fractions, membranes, nuclei, mitochondria, and cytoskeleton reviewed in (Tuazon and Traugh, *Adv. Second Messenger Phosphoprotein Res.*, 1991, 23, 123-164).

Casein kinase 2 (also known generally as casein kinase  
20 as well as CKII, CK2 and CSNK2) is a heterotetrameric holoenzyme composed of two catalytic (alpha and/or alpha prime (alpha')) subunits (Lozeman et al., *Biochemistry*, 1990, 29, 8436-8447; Meisner et al., *Biochemistry*, 1989, 28, 4072-4076) and two regulatory beta subunits (Heller-Harrison  
25 et al., *Biochemistry*, 1989, 28, 9053-9058; Jakobi et al., *Eur. J. Biochem.*, 1989, 183, 227-233). While the alpha subunits contain the enzyme's active site, the noncatalytic beta subunit functions to protect the alpha subunit against denaturing agents or conditions and alters the substrate  
30 specificity of the enzyme. The alpha subunit protein has also been shown to activate transcription of the beta subunit gene (Robitzki et al., *J. Biol. Chem.*, 1993, 268, 5694-5702).

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Casein kinase 2 is unique in that it recognizes phosphoacceptor sites specified by several acidic determinants, it can use both ATP and GTP as phosphoryl donors, it is insensitive to any known second messenger and displays high basal activity.

Casein kinase 2 is constitutively active, highly pleiotropic and its targeting seems to be modulated through association with a variety of cellular proteins, more than 160 of which are known (Pinna and Meggio, *Prog. Cell Cycle Res.*, **1997**, 3, 77-97). Its expression is abnormally elevated in proliferating and neoplastic tissues and recent studies suggest that mice overexpressing the alpha subunit of casein kinase 2 develop leukemia. Several compounds have been shown to affect the activity of casein kinase 2 with polycationic species stimulating activity and polyanionic species acting as inhibitors (Pinna, *Biochim. Biophys. Acta*, **1990**, 1054, 267-284).

Physiologically, casein kinase 2 has been shown to play a role in cell cycle progression (Marshak and Russo, *Cell. Mol. Biol. Res.*, **1994**, 40, 513-517), liver regeneration (Perez et al., *FEBS Lett.*, **1988**, 238, 273-276), viral replication (Dupuy et al., *J. Virol.*, **1999**, 73, 8384-8392; Lenard, *Pharmacol. Ther.*, **1999**, 83, 39-48; Schubert et al., *J. Mol. Biol.*, **1994**, 236, 16-25; Wadd et al., *J. Biol. Chem.*, **1999**, 274, 28991-28998), apoptosis (Watabe et al., *Cell. Growth Differ.*, **1997**, 8, 871-879), transduction of growth signals (Tawfic and Ahmed, *J. Biol. Chem.*, **1994**, 269, 24615-24620), prostate cancer (Yenice et al., *Prostate*, **1994**, 24, 11-16), breast cancer (O'Brien et al., *Biochem. Biophys. Res. Commun.*, **1999**, 260, 658-664), insulin signaling (Singh, *Mol. Cell. Biochem.*, **1993**, 121, 167-174) and Alzheimer's disease (Blanquet, *Neuroscience*, **1998**, 86, 739-749).

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Inhibitors of the enzyme, as a whole or subunit-specific, may have therapeutic potential and consequently, modulation of casein kinase 2 activity and/or expression is believed to be an appropriate point of therapeutic  
5 intervention in pathological conditions.

To date, investigative strategies aimed at modulating casein kinase 2 function have involved the use of antibodies, antisense oligonucleotides, and chemical inhibitors.

10 Disclosed in U.S. Patent 5,171,217 are methods of delivering inhibitors of protein kinases, including casein kinase 2, to an affected intramural site (March et al., 1992).

Antisense oligonucleotides targeting each of the  
15 subunits of casein kinase 2 in various organisms have been reported in the art. Chen et al. designed one antisense oligonucleotide targeting the beta subunit of casein kinase 2 in *Xenopus* to investigate oocyte maturation (Chen and Cooper, *Proc. Natl. Acad. Sci. U. S. A.*, 1997, 94, 9136-  
20 9140). This oligonucleotide targeted residues 175-182 and contained phosphodiester links with the three phosphodiester links at the 3' end replaced by phosphorothioate links.

Shayan et al. report the use of an antisense oligonucleotide targeting the alpha subunit of casein kinase  
25 2 in viral infected bovine lymphoblastoid cells (Shayan and Ahmed, *Parasitol. Res.*, 1997, 83, 526-532). In the mouse, antisense oligonucleotides targeting the start codon of each of the three subunits have been designed and used to investigate the process of neuritogenesis (Ulloa et al.,  
30 *Embo J.*, 1993, 12, 1633-1640; Ulloa et al., *Cell. Mol. Neurobiol.*, 1994, 14, 407-414).

Antisense oligonucleotides, targeting the start codon of each of the subunits of human casein kinase 2, have also

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been reported (Pepperkok et al., *Exp. Cell. Res.*, **1991**, 197, 245-253; Pyerin et al., *Ann. N. Y. Acad. Sci.*, **1992**, 660, 295-297). In these studies of human fibroblasts (IMR-90 cells), antisense oligonucleotides were used to investigate the role of casein kinase 2 in early growth stimulation by epidermal growth factor (Pepperkok et al., *Exp. Cell. Res.*, **1991**, 197, 245-253; Pyerin et al., *Ann. N. Y. Acad. Sci.*, **1992**, 660, 295-297).

In MDA231 breast carcinoma cell lines, an antisense oligonucleotide targeting the start codon of the alpha prime subunit of human casein kinase 2 was used to investigate alternative splicing patterns in the CD44 gene (Formby and Stern, *Mol. Cell. Biochem.*, **1998**, 187, 23-31). And in human Ca9-22 cells derived from squamous cell carcinomas of the head and neck (SCCHN), transfection with a phosphodiester antisense oligonucleotide targeting the alpha subunit of human casein kinase 2 resulted in growth inhibition of the carcinoma cell line (Faust et al., *Head Neck*, **2000**, 22, 341-346).

Antisense technology is emerging as an effective means for reducing the expression of specific gene products and may therefore prove to be uniquely useful in a number of therapeutic, diagnostic, and research applications for the modulation of casein kinase 2 expression.

The present invention provides compositions and methods for modulating the expression of the beta subunit of casein kinase 2.

#### **SUMMARY OF THE INVENTION**

The present invention is directed to compounds, particularly antisense oligonucleotides, which are targeted to a nucleic acid encoding Casein kinase 2-beta, and which modulate the expression of Casein kinase 2-beta. Pharmaceutical and other compositions comprising the

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compounds of the invention are also provided. Further provided are methods of modulating the expression of Casein kinase 2-beta in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds  
5 or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of Casein kinase 2-beta by administering a therapeutically or prophylactically  
10 effective amount of one or more of the antisense compounds or compositions of the invention.

**DETAILED DESCRIPTION OF THE INVENTION**

The present invention employs oligomeric compounds, particularly antisense oligonucleotides, for use in  
15 modulating the function of nucleic acid molecules encoding Casein kinase 2-beta, ultimately modulating the amount of Casein kinase 2-beta produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding Casein kinase 2-  
20 beta. As used herein, the terms "target nucleic acid" and "nucleic acid encoding Casein kinase 2-beta" encompass DNA encoding Casein kinase 2-beta, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric  
25 compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with  
30 include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing

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of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of Casein kinase 2-beta. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. In the context of the present invention, inhibition is the preferred form of modulation of gene expression and mRNA is a preferred target.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding Casein kinase 2-beta. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a



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translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function in vivo. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon  
5 sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be  
10 preferentially utilized for translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an  
15 mRNA molecule transcribed from a gene encoding Casein kinase 2-beta, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the  
20 corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or  
25 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation  
30 termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination

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codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

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Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired  
5 effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and  
10 thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen  
15 bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of  
20 corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of  
25 complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is  
30 specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity

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to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment, and in  
5 the case of in vitro assays, under conditions in which the assays are performed.

Antisense and other compounds of the invention which hybridize to the target and inhibit expression of the target are identified through experimentation, and the sequences of  
10 these compounds are hereinbelow identified as preferred embodiments of the invention. The target sites to which these preferred sequences are complementary are hereinbelow referred to as "active sites" and are therefore preferred sites for targeting. Therefore another embodiment of the  
15 invention encompasses compounds which hybridize to these active sites.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression  
20 with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore,  
25 been harnessed for research use.

For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other antisense compounds or therapeutics, can be used as tools in differential and/or combinatorial  
30 analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues.

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Expression patterns within cells or tissues treated with one or more antisense compounds are compared to control cells or tissues not treated with antisense compounds and the patterns produced are analyzed for differential levels of gene expression as they pertain, for example, to disease association, signaling pathway, cellular localization, expression level, size, structure or function of the genes examined. These analyses can be performed on stimulated or unstimulated cells and in the presence or absence of other compounds which affect expression patterns.

Examples of methods of gene expression analysis known in the art include DNA arrays or microarrays (Brazma and Vilo, *FEBS Lett.*, **2000**, 480, 17-24; Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16), SAGE (serial analysis of gene expression) (Madden, et al., *Drug Discov. Today*, **2000**, 5, 415-425), READS (restriction enzyme amplification of digested cDNAs) (Prashar and Weissman, *Methods Enzymol.*, **1999**, 303, 258-72), TOGA (total gene expression analysis) (Sutcliffe, et al., *Proc. Natl. Acad. Sci. U. S. A.*, **2000**, 97, 1976-81), protein arrays and proteomics (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Jungblut, et al., *Electrophoresis*, **1999**, 20, 2100-10), expressed sequence tag (EST) sequencing (Celis, et al., *FEBS Lett.*, **2000**, 480, 2-16; Larsson, et al., *J. Biotechnol.*, **2000**, 80, 143-57), subtractive RNA fingerprinting (SuRF) (Fuchs, et al., *Anal. Biochem.*, **2000**, 286, 91-98; Larson, et al., *Cytometry*, **2000**, 41, 203-208), subtractive cloning, differential display (DD) (Jurecic and Belmont, *Curr. Opin. Microbiol.*, **2000**, 3, 316-21), comparative genomic hybridization (Carulli, et al., *J. Cell Biochem. Suppl.*, **1998**, 31, 286-96), FISH (fluorescent in situ hybridization) techniques (Going and Gusterson, *Eur. J. Cancer*, **1999**, 35, 1895-904) and mass spectrometry methods

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(reviewed in (To, *Comb. Chem. High Throughput Screen*, 2000, 3, 235-41).

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotide drugs, including ribozymes, have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 50 nucleobases (i.e. from about 8 to about 50 linked nucleosides). Particularly preferred antisense compounds

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are antisense oligonucleotides, even more preferably those comprising from about 12 to about 30 nucleobases. Antisense compounds include ribozymes, external guide sequence (EGS) oligonucleotides (oligozymes), and other short catalytic  
5 RNAs or catalytic oligonucleotides which hybridize to the target nucleic acid and modulate its expression.

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such  
10 heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to  
15 either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a  
20 circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5'  
25 phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having  
30 modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art,

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modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates, 5'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, selenophosphates and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein one or more internucleotide linkages is a 3' to 3', 5' to 5' or 2' to 2' linkage. Preferred oligonucleotides having inverted polarity comprise a single 3' to 3' linkage at the 3'-most internucleotide linkage i.e. a single inverted nucleoside residue which may be abasic (the nucleobase is missing or has a hydroxyl group in place thereof). Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S.: 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,194,599; 5,565,555; 5,527,899; 5,721,218; 5,672,697 and 5,625,050, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.



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Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; riboacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH<sub>2</sub> component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S.: 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,792,608; 5,646,269 and 5,677,439, certain of which are commonly owned with this application, and each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is

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referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S.: 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, 254, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular  $-\text{CH}_2-\text{NH}-\text{O}-\text{CH}_2-$ ,  $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$  [known as a methylene (methylinino) or MMI backbone],  $-\text{CH}_2-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-$ ,  $-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)-\text{CH}_2-$  and  $-\text{O}-\text{N}(\text{CH}_3)-\text{CH}_2-\text{CH}_2-$  [wherein the native phosphodiester backbone is represented as  $-\text{O}-\text{P}-\text{O}-\text{CH}_2-$ ] of the above referenced U.S. patent 5,489,677, and the amide backbones of the above referenced U.S. patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted  $\text{C}_1$  to  $\text{C}_{10}$  alkyl or  $\text{C}_2$  to  $\text{C}_{10}$  alkenyl and alkynyl. Particularly preferred are  $\text{O}[(\text{CH}_2)_n\text{O}]_m\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{OCH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{NH}_2$ ,  $\text{O}(\text{CH}_2)_n\text{CH}_3$ ,  $\text{O}(\text{CH}_2)_n\text{ONH}_2$ , and  $\text{O}(\text{CH}_2)_n\text{ON}[(\text{CH}_2)_n\text{CH}_3]_2$ , where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the

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following at the 2' position: C<sub>1</sub> to C<sub>10</sub> lower alkyl, substituted lower alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH<sub>3</sub>, OCN, Cl, Br, CN, CF<sub>3</sub>, OCF<sub>3</sub>, SOCH<sub>3</sub>, SO<sub>2</sub>CH<sub>3</sub>, ONO<sub>2</sub>, NO<sub>2</sub>, N<sub>3</sub>, NH<sub>2</sub>, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O-CH<sub>2</sub>CH<sub>2</sub>OCH<sub>3</sub>, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, 78, 486-504) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminoethoxyethoxy, i.e., a O(CH<sub>2</sub>)<sub>2</sub>ON(CH<sub>3</sub>)<sub>2</sub> group, also known as 2'-DMAOE, as described in examples hereinbelow, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>3</sub>)<sub>2</sub>, also described in examples hereinbelow.

A further preferred modification includes Locked Nucleic Acids (LNAs) in which the 2'-hydroxyl group is linked to the 3' or 4' carbon atom of the sugar ring thereby forming a bicyclic sugar moiety. The linkage is preferably a methylene (-CH<sub>2</sub>-)<sub>n</sub> group bridging the 2' oxygen atom and the 4' carbon atom wherein n is 1 or 2. LNAs and preparation thereof are described in WO 98/39352 and WO 99/14226.

Other preferred modifications include 2'-methoxy (2'-O-CH<sub>3</sub>), 2'-aminopropoxy (2'-OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>NH<sub>2</sub>), 2'-allyl (2'-CH<sub>2</sub>-CH=CH<sub>2</sub>), 2'-O-allyl (2'-O-CH<sub>2</sub>-CH=CH<sub>2</sub>) and 2'-fluoro (2'-F). The 2'-modification may be in the arabino (up) position or ribo (down) position. A preferred 2'-arabino modification is 2'-F. Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3'

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position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 5,792,747; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl ( $-C\equiv C-CH_3$ ) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-

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amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further modified nucleobases include tricyclic pyrimidines such as phenoxazine cytidine (1H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H-pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps such as a substituted phenoxazine cytidine (e.g. 9-(2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5-b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one). Modified nucleobases may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Further nucleobases include those disclosed in United States Patent No. 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y.S., Chapter 15, *Antisense Research and Applications*, pages 289-302, Crooke, S.T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently

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preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified  
5 nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. 3,687,808, as well as U.S.: 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121,  
10 5,596,091; 5,614,617; 5,645,985; 5,830,653; 5,763,588; 6,005,096; and 5,681,941, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference, and United States patent 5,750,692, which is commonly owned with the instant  
15 application and also herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the  
20 oligonucleotide. The compounds of the invention can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers,  
25 groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugates groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluores-  
30 ceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve oligomer uptake, enhance oligomer resistance to degradation, and/or

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strengthen sequence-specific hybridization with RNA. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve oligomer uptake, distribution, metabolism or excretion.

- 5 Representative conjugate groups are disclosed in International Patent Application PCT/US92/09196, filed October 23, 1992 the entire disclosure of which is incorporated herein by reference. Conjugate moieties include but are not limited to lipid moieties such as a cholesterol
- 10 moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Lett.*,
- 15 **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330; Svinarchuk et al., *Biochimie*,
- 20 **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain
- 25 (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-
- 30 oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937. Oligonucleotides of the invention may also be conjugated to active drug substances, for example, aspirin, warfarin, phenylbutazone, ibuprofen,

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suprofen, fenbufen, ketoprofen, (S) - (+) -pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indomethicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic. Oligonucleotide-drug conjugates and their preparation are described in United States Patent Application 09/334,130 (filed June 15, 1999) which is incorporated herein by reference in its entirety.

10        Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S.: 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 15 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 20 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, certain of which are commonly owned with the 25 instant application, and each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. 30 "Chimeric" antisense compounds or "chimeras," in the context



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of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S.: 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and

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5,700,922, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized in vitro and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the in vivo synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S.:

5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291;  
5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330;  
4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170;  
5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978;  
5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259;  
5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

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The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing  
5 (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and  
10 other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or  
15 conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published December 9, 1993 or in WO 94/26764 and U.S.  
20 5,770,713 to Imbach et al.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do  
25 not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the  
30 like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et

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al., "Pharmaceutical Salts," *J. of Pharma Sci.*, 1977, 66, 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a "pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred acid salts are the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylemaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embonic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in

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nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfonic acid, 5 naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of 10 compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth, ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are 15 also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as 20 spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric 25 acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, 30 polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as

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research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of Casein kinase 2-beta is treated by administering  
5 antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of  
10 the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize  
15 to nucleic acids encoding Casein kinase 2-beta, enabling sandwich and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding Casein kinase 2-beta can be detected by means known  
20 in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of Casein kinase 2-beta in a sample may also be prepared.

25 The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is  
30 desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including

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by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or  
5 intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for  
10 topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves  
15 and the like may also be useful. Preferred topical formulations include those in which the oligonucleotides of the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Preferred  
20 lipids and liposomes include neutral (e.g. dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative (e.g. dimyristoylphosphatidyl glycerol DMPG) and cationic (e.g. dioleoyltetramethylaminopropyl  
25 DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). Oligonucleotides of the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, oligonucleotides may be complexed to lipids, in particular to cationic lipids.  
30 Preferred fatty acids and esters include but are not limited arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate,

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tricaprates, monoolein, dilaurin, glyceryl 1-monocaprates, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C<sub>1-10</sub> alkyl ester (e.g. isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in United States patent application 09/315,298 filed on May 20, 1999 which is incorporated herein by reference in its entirety.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitables. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate, . Preferred fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprates, tricaprates, monoolein, dilaurin, glyceryl 1-monocaprates, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of



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penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include

5 polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include

10 poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines,

15 pollulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino),

20 poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-

25 lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for oligonucleotides and their preparation are described in detail in United States applications 08/886,829 (filed July 1, 1997), 09/108,673 (filed July 1, 1998), 09/256,515 (filed

30 February 23, 1999), 09/082,624 (filed May 21, 1998) and 09/315,298 (filed May 20, 1999) each of which is incorporated herein by reference in their entirety.

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Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not  
5 limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be  
10 generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit  
15 dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general the formulations are prepared by  
20 uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be  
25 formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media.  
30 Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

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In one embodiment of the present invention the pharmaceutical compositions may be formulated and used as foams. Pharmaceutical foams include formulations such as, but not limited to, emulsions, microemulsions, creams, 5 jellies and liposomes. While basically similar in nature these formulations vary in the components and the consistency of the final product. The preparation of such compositions and formulations is generally known to those skilled in the pharmaceutical and formulation arts and may 10 be applied to the formulation of the compositions of the present invention.

#### Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are 15 typically heterogenous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1  $\mu\text{m}$  in diameter. (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in 20 *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi et al., in *Remington's* 25 *Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 301). Emulsions are often biphasic systems comprising of two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be either water-in-oil (w/o) or of the oil-in-water (o/w) 30 variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely

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divided into and dispersed as minute droplets into a bulk aqueous phase the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

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Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

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A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents

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such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of reasons of ease of formulation, efficacy from an absorption and bioavailability standpoint. (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of oligonucleotides and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described

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as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: *Controlled Release of Drugs: Polymers and Aggregate Systems*, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in *Remington's Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in *Pharmaceutical Dosage Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate



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(MO750), decaglycerol sequeioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides et al., *Pharmaceutical Research*, **1994**, *11*, 1385-1390; Ritschel, *Meth. Find. Exp. Clin. Pharmacol.*, **1993**, *13*, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical

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potency, and decreased toxicity (Constantinides et al.,  
*Pharmaceutical Research*, 1994, 11, 1385; Ho et al., *J.*  
*Pharm. Sci.*, 1996, 85, 138-143). Often microemulsions may  
form spontaneously when their components are brought  
5 together at ambient temperature. This may be particularly  
advantageous when formulating thermolabile drugs, peptides  
or oligonucleotides. Microemulsions have also been  
effective in the transdermal delivery of active components  
in both cosmetic and pharmaceutical applications. It is  
10 expected that the microemulsion compositions and  
formulations of the present invention will facilitate the  
increased systemic absorption of oligonucleotides and  
nucleic acids from the gastrointestinal tract, as well as  
improve the local cellular uptake of oligonucleotides and  
15 nucleic acids within the gastrointestinal tract, vagina,  
buccal cavity and other areas of administration.

Microemulsions of the present invention may also  
contain additional components and additives such as sorbitan  
monostearate (Grill 3), Labrasol, and penetration enhancers  
20 to improve the properties of the formulation and to enhance  
the absorption of the oligonucleotides and nucleic acids of  
the present invention. Penetration enhancers used in the  
microemulsions of the present invention may be classified as  
belonging to one of five broad categories - surfactants,  
25 fatty acids, bile salts, chelating agents, and non-chelating  
non-surfactants (Lee et al., *Critical Reviews in Therapeutic*  
*Drug Carrier Systems*, 1991, p. 92). Each of these classes  
has been discussed above.

#### Liposomes

30 There are many organized surfactant structures besides  
microemulsions that have been studied and used for the  
formulation of drugs. These include monolayers, micelles,  
bilayers and vesicles. Vesicles, such as liposomes, have

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attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids  
5 arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the  
10 advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a  
15 diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes  
20 obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in *Pharmaceutical Dosage*  
25 *Forms*, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

30 Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the

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liposomes start to merge with the cellular membranes. As the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

5        Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects  
10 related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

      Several reports have detailed the ability of liposomes  
15 to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis.

20        Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized  
25 in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang et al., *Biochem. Biophys. Res. Commun.*, **1987**, *147*, 980-985).

      Liposomes which are pH-sensitive or negatively-charged,  
30 entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes.

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pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou et al., *Journal of Controlled Release*, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (e.g. as a solution or as an emulsion) were ineffective (Weiner et al., *Journal of Drug Targeting*, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis et al., *Antiviral Research*, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations

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comprising Novasome™ I (glyceryl  
dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and  
Novasome™ II (glyceryl distearate/  
cholesterol/polyoxyethylene-10-stearyl ether) were used to  
5 deliver cyclosporin-A into the dermis of mouse skin.  
Results indicated that such non-ionic liposomal systems were  
effective in facilitating the deposition of cyclosporin-A  
into different layers of the skin (Hu et al. *S.T.P. Pharma.*  
*Sci.*, 1994, 4, 6, 466).

10       Liposomes also include "sterically stabilized"  
liposomes, a term which, as used herein, refers to liposomes  
comprising one or more specialized lipids that, when  
incorporated into liposomes, result in enhanced circulation  
lifetimes relative to liposomes lacking such specialized  
15 lipids. Examples of sterically stabilized liposomes are  
those in which part of the vesicle-forming lipid portion of  
the liposome (A) comprises one or more glycolipids, such as  
monosialoganglioside G<sub>M1</sub>, or (B) is derivatized with one or  
more hydrophilic polymers, such as a polyethylene glycol  
20 (PEG) moiety. While not wishing to be bound by any  
particular theory, it is thought in the art that, at least  
for sterically stabilized liposomes containing gangliosides,  
sphingomyelin, or PEG-derivatized lipids, the enhanced  
circulation half-life of these sterically stabilized  
25 liposomes derives from a reduced uptake into cells of the  
reticuloendothelial system (RES) (Allen et al., *FEBS*  
*Letters*, 1987, 223, 42; Wu et al., *Cancer Research*, 1993,  
53, 3765). Various liposomes comprising one or more  
glycolipids are known in the art. Papahadjopoulos et al.  
30 (*Ann. N.Y. Acad. Sci.*, 1987, 507, 64) reported the ability  
of monosialoganglioside G<sub>M1</sub>, galactocerebroside sulfate and  
phosphatidylinositol to improve blood half-lives of  
liposomes. These findings were expounded upon by Gabizon et

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al. (*Proc. Natl. Acad. Sci. U.S.A.*, 1988, 85, 6949). U.S. Patent No. 4,837,028 and WO 88/04924, both to Allen et al., disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G<sub>M1</sub> or a galactocerebroside sulfate ester. U.S. Patent No. 5,543,152 (Webb et al.) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-*sn*-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim et al.).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto et al. (*Bull. Chem. Soc. Jpn.*, 1980, 53, 2778) described liposomes comprising a nonionic detergent, 2C<sub>12</sub>15G, that contains a PEG moiety. Illum et al. (*FEBS Lett.*, 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (e.g., PEG) are described by Sears (U.S. Patent Nos. 4,426,330 and 4,534,899). Klibanov et al. (*FEBS Lett.*, 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume et al. (*Biochimica et Biophysica Acta*, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, e.g., DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle et al. (U.S.

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Patent Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Patent No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Patent No. 5,225,212 (both to Martin *et al.*) and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al.*). U.S. Patent Nos. 5,540,935 (Miyazaki *et al.*) and 5,556,948 (Tagawa *et al.*) describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A limited number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Patent No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include an antisense RNA. U.S. Patent No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising antisense oligonucleotides targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, e.g. they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators,



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usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection  
5 of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and  
10 synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York,  
15 NY, 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In  
20 general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic  
25 alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

30 If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of

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amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members  
5 of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include  
10 quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is  
15 classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in  
20 *Pharmaceutical Dosage Forms*, Marcel Dekker, Inc., New York, NY, 1988, p. 285).

#### Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient  
25 delivery of nucleic acids, particularly oligonucleotides, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic  
30 drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell

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membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, i.e., surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

10        Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another  
15 liquid, with the result that absorption of oligonucleotides through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee et al.,  
20 *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi et al., *J. Pharm. Pharmacol.*, 1988, 40, 252).

      Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example,  
25 oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-  
30 dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C<sub>1-10</sub> alkyl esters thereof (e.g., methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate,

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etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, p.92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, 1990, 7, 1-33; El Hariri et al., *J. Pharm. Pharmacol.*, 1992, 44, 651-654).

- 5 Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's *The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al. Eds., McGraw-Hill, New York, 1996, pp. 934-935).
- 10 Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. The bile salts of the invention include, for example, cholic acid (or
- 15 its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucolic acid (sodium glucolate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate),
- 20 taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether
- 25 (POE) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, 1991, page 92; Swinyard, Chapter 39 In: *Remington's Pharmaceutical Sciences*, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, PA, 1990, pages 782-783; Muranishi, *Critical Reviews in Therapeutic Drug Carrier*
- 30 *Systems*, 1990, 7, 1-33; Yamamoto et al., *J. Pharm. Exp. Ther.*, 1992, 263, 25; Yamashita et al., *J. Pharm. Sci.*, 1990, 79, 579-583).

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Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of oligonucleotides through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, *J. Chromatogr.*, **1993**, *618*, 315-339). Chelating agents of the invention include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), *N*-acyl derivatives of collagen, laureth-9 and *N*-amino acyl derivatives of beta-diketones (enamines) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33; Buur et al., *J. Control Rel.*, **1990**, *14*, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of oligonucleotides through the alimentary mucosa (Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, *7*, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacycloalkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, page 92); and non-steroidal anti-inflammatory agents such as diclofenac

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sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, **1987**, 39, 621-626).

Agents that enhance uptake of oligonucleotides at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi et al, U.S. Patent No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo et al., PCT Application WO 97/30731), are also known to enhance the cellular uptake of oligonucleotides.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

#### 15 Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity *per se*) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate oligonucleotide in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate,

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polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, 1995, 5, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, 1996, 6, 177-183).

## 5 Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an  
10 animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical  
15 pharmaceutical carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium  
20 sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium  
25 acetate, etc.); disintegrants (e.g., starch, sodium starch glycolate, etc.); and wetting agents (e.g., sodium lauryl sulphate, etc.).

Pharmaceutically acceptable organic or inorganic excipient suitable for non-parenteral administration which  
30 do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols,

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polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

#### Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can



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be sterilized and, if desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances  
5 and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or  
10 dextran. The suspension may also contain stabilizers.

Certain embodiments of the invention provide pharmaceutical compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense  
15 mechanism. Examples of such chemotherapeutic agents include but are not limited to daunorubicin, daunomycin, dactinomycin, doxorubicin, epirubicin, idarubicin, esorubicin, bleomycin, mafosfamide, ifosfamide, cytosine arabinoside, bis-chloroethylnitrosurea, busulfan, mitomycin  
20 C, actinomycin D, mithramycin, prednisone, hydroxyprogesterone, testosterone, tamoxifen, dacarbazine, procarbazine, hexamethylmelamine, pentamethylmelamine, mitoxantrone, amsacrine, chlorambucil, methylcyclohexylnitrosurea, nitrogen mustards, melphalan,  
25 cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-azacytidine, hydroxyurea, deoxycoformycin, 4-hydroxyperoxycyclophosphoramide, 5-fluorouracil (5-FU), 5-fluorodeoxyuridine (5-FUdR), methotrexate (MTX), colchicine, taxol, vincristine, vinblastine, etoposide (VP-16),  
30 trimetrexate, irinotecan, topotecan, gemcitabine, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed. 1987, pp. 1206-1228, Berkow et al., eds., Rahway, N.J.

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When used with the compounds of the invention, such  
chemotherapeutic agents may be used individually (e.g., 5-FU  
and oligonucleotide), sequentially (e.g., 5-FU and  
oligonucleotide for a period of time followed by MTX and  
5 oligonucleotide), or in combination with one or more other  
such chemotherapeutic agents (e.g., 5-FU, MTX and  
oligonucleotide, or 5-FU, radiotherapy and oligonucleotide).  
Anti-inflammatory drugs, including but not limited to  
nonsteroidal anti-inflammatory drugs and corticosteroids,  
10 and antiviral drugs, including but not limited to ribivirin,  
vidarabine, acyclovir and ganciclovir, may also be combined  
in compositions of the invention. See, generally, *The Merck  
Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al.,  
eds., 1987, Rahway, N.J., pages 2499-2506 and 46-49,  
15 respectively). Other non-antisense chemotherapeutic agents  
are also within the scope of this invention. Two or more  
combined compounds may be used together or sequentially.

In another related embodiment, compositions of the  
invention may contain one or more antisense compounds,  
20 particularly oligonucleotides, targeted to a first nucleic  
acid and one or more additional antisense compounds targeted  
to a second nucleic acid target. Numerous examples of  
antisense compounds are known in the art. Two or more  
combined compounds may be used together or sequentially.

25 The formulation of therapeutic compositions and their  
subsequent administration is believed to be within the skill  
of those in the art. Dosing is dependent on severity and  
responsiveness of the disease state to be treated, with the  
course of treatment lasting from several days to several  
30 months, or until a cure is effected or a diminution of the  
disease state is achieved. Optimal dosing schedules can be  
calculated from measurements of drug accumulation in the  
body of the patient. Persons of ordinary skill can easily

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determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC<sub>50</sub>s found to be effective in in vitro and in vivo animal models. In general, dosage is from 0.01 ug to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 ug to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

#### EXAMPLES

##### Example 1

##### Nucleoside Phosphoramidites for Oligonucleotide Synthesis Deoxy and 2'-alkoxy amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after

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pulse delivery of tetrazole and base was increased to 360 seconds.

Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published  
5 methods [Sanghvi, et. al., *Nucleic Acids Research*, 1993, 21, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

#### **2'-Fluoro amidites**

##### **2'-Fluorodeoxyadenosine amidites**

10 2'-fluoro oligonucleotides were synthesized as described previously [Kawasaki, et. al., *J. Med. Chem.*, 1993, 36, 831-841] and United States patent 5,670,633, herein incorporated by reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was  
15 synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S<sub>N</sub>2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was  
20 selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite  
25 intermediates.

##### **2'-Fluorodeoxyguanosine**

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS)  
protected 9-beta-D-arabinofuranosylguanine as starting  
30 material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine.

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Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

5        **2'-Fluorouridine**

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used  
10 to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

**2'-Fluorodeoxycytidine**

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-  
15 fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

**2'-O-(2-Methoxyethyl) modified amidites**

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of  
20 Martin, P., *Helvetica Chimica Acta*, 1995, 78, 486-504.

**2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]**

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-  
25 carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under  
30 reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution

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was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions (or it can be purified further by column chromatography using a gradient of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C).

**2'-O-Methoxyethyl-5-methyluridine**

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH<sub>3</sub>CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH<sub>2</sub>Cl<sub>2</sub>/acetone/MeOH (20:5:3) containing 0.5% Et<sub>3</sub>NH. The residue was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the

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mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC  
5 showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH<sub>3</sub>CN (200 mL). The residue was dissolved in CHCl<sub>3</sub> (1.5 L) and extracted with 2x500 mL of saturated NaHCO<sub>3</sub> and 2x500 mL of saturated NaCl. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>, filtered and  
10 evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/hexane/acetone (5:5:1) containing 0.5% Et<sub>3</sub>NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure  
15 fractions to give a total yield of 183 g (57%).

**3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture  
20 prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by TLC by first quenching the TLC sample with the addition of MeOH. Upon completion of the reaction, as  
25 judged by TLC, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in CHCl<sub>3</sub> (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of CHCl<sub>3</sub>. The  
30 combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/hexane(4:1). Pure product fractions were

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evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

**3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine**

5 A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH<sub>3</sub>CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH<sub>3</sub>CN (1 L), cooled to  
10 -5°C and stirred for 0.5 h using an overhead stirrer. POCl<sub>3</sub> was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter  
15 solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed  
20 with 1x300 mL of NaHCO<sub>3</sub> and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

**2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M)  
25 in dioxane (500 mL) and NH<sub>4</sub>OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred  
30 to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH<sub>3</sub> gas was added and the vessel heated to 100°C for 2 hours (TLC showed complete conversion). The vessel contents were evaporated to dryness and the residue



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was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

5        **N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine**

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After  
10 stirring for 3 hours, TLC showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl<sub>3</sub> (700 mL) and extracted with saturated NaHCO<sub>3</sub> (2x300 mL) and saturated NaCl (2x300 mL), dried over  
15 MgSO<sub>4</sub> and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/hexane (1:1) containing 0.5% Et<sub>3</sub>NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

20        **N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite**

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-  
25 (isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (TLC showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO<sub>3</sub> (1x300 mL) and  
30 saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH<sub>2</sub>Cl<sub>2</sub> (300 mL), and the extracts were combined, dried over MgSO<sub>4</sub> and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using

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EtOAc/hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

**2'-O-(Aminooxyethyl) nucleoside amidites and 2'-O-**

5 **(dimethylaminooxyethyl) nucleoside amidites**

**2'-(Dimethylaminooxyethoxy) nucleoside amidites**

2'-(Dimethylaminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(dimethylaminooxyethyl) nucleoside amidites] are prepared as described in the following  
10 paragraphs. Adenosine, cytidine and guanosine nucleoside amidites are prepared similarly to the thymidine (5-methyluridine) except the exocyclic amines are protected with a benzoyl moiety in the case of adenosine and cytidine and with isobutyryl in the case of guanosine.

15 **5'-O-tert-Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine**

O<sup>2</sup>-2'-anhydro-5-methyluridine (Pro. Bio. Sint., Varese, Italy, 100.0g, 0.416 mmol), dimethylaminopyridine (0.66g, 0.013eq, 0.0054mmol) were dissolved in dry pyridine (500 ml)  
20 at ambient temperature under an argon atmosphere and with mechanical stirring. tert-Butyldiphenylchlorosilane (125.8g, 119.0mL, 1.1eq, 0.458mmol) was added in one portion. The reaction was stirred for 16 h at ambient temperature. TLC (Rf 0.22, ethyl acetate) indicated a  
25 complete reaction. The solution was concentrated under reduced pressure to a thick oil. This was partitioned between dichloromethane (1 L) and saturated sodium bicarbonate (2x1 L) and brine (1 L). The organic layer was dried over sodium sulfate and concentrated under reduced  
30 pressure to a thick oil. The oil was dissolved in a 1:1 mixture of ethyl acetate and ethyl ether (600mL) and the solution was cooled to -10°C. The resulting crystalline product was collected by filtration, washed with ethyl ether

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(3x200 mL) and dried (40°C, 1mm Hg, 24 h) to 149g (74.8%) of white solid. TLC and NMR were consistent with pure product.

**5'-O-tert-Butyldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine**

5 In a 2 L stainless steel, unstirred pressure reactor was added borane in tetrahydrofuran (1.0 M, 2.0 eq, 622 mL). In the fume hood and with manual stirring, ethylene glycol (350 mL, excess) was added cautiously at first until the evolution of hydrogen gas subsided. 5'-O-tert-

10 Butyldiphenylsilyl-O<sup>2</sup>-2'-anhydro-5-methyluridine (149 g, 0.311 mol) and sodium bicarbonate (0.074 g, 0.003 eq) were added with manual stirring. The reactor was sealed and heated in an oil bath until an internal temperature of 160 °C was reached and then maintained for 16 h (pressure < 100

15 psig). The reaction vessel was cooled to ambient and opened. TLC (Rf 0.67 for desired product and Rf 0.82 for ara-T side product, ethyl acetate) indicated about 70% conversion to the product. In order to avoid additional side product formation, the reaction was stopped,

20 concentrated under reduced pressure (10 to 1mm Hg) in a warm water bath (40-100°C) with the more extreme conditions used to remove the ethylene glycol. [Alternatively, once the low boiling solvent is gone, the remaining solution can be partitioned between ethyl acetate and water. The product

25 will be in the organic phase.] The residue was purified by column chromatography (2kg silica gel, ethyl acetate-hexanes gradient 1:1 to 4:1). The appropriate fractions were combined, stripped and dried to product as a white crisp foam (84g, 50%), contaminated starting material (17.4g) and

30 pure reusable starting material 20g. The yield based on starting material less pure recovered starting material was 58%. TLC and NMR were consistent with 99% pure product.

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**2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylldiphenylsilyl-5-methyluridine**

5'-O-tert-Butylldiphenylsilyl-2'-O-(2-hydroxyethyl)-5-methyluridine (20g, 36.98mmol) was mixed with triphenylphosphine (11.63g, 44.36mmol) and N-hydroxyphthalimide (7.24g, 44.36mmol). It was then dried over P<sub>2</sub>O<sub>5</sub> under high vacuum for two days at 40°C. The reaction mixture was flushed with argon and dry THF (369.8mL, Aldrich, sure seal bottle) was added to get a clear solution. Diethyl-azodicarboxylate (6.98mL, 44.36mmol) was added dropwise to the reaction mixture. The rate of addition is maintained such that resulting deep red coloration is just discharged before adding the next drop. After the addition was complete, the reaction was stirred for 4 hrs. By that time TLC showed the completion of the reaction (ethylacetate:hexane, 60:40). The solvent was evaporated in vacuum. Residue obtained was placed on a flash column and eluted with ethyl acetate:hexane (60:40), to get 2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylldiphenylsilyl-5-methyluridine as white foam (21.819 g, 86%).

**5'-O-tert-butylldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine**

2'-O-([2-phthalimidoxy)ethyl]-5'-t-butylldiphenylsilyl-5-methyluridine (3.1g, 4.5mmol) was dissolved in dry CH<sub>2</sub>Cl<sub>2</sub> (4.5mL) and methylhydrazine (300mL, 4.64mmol) was added dropwise at -10°C to 0°C. After 1 h the mixture was filtered, the filtrate was washed with ice cold CH<sub>2</sub>Cl<sub>2</sub> and the combined organic phase was washed with water, brine and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>. The solution was concentrated to get 2'-O-(aminooxyethyl) thymidine, which was then dissolved in MeOH (67.5mL). To this formaldehyde (20% aqueous solution, w/w, 1.1 eq.) was added and the resulting

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mixture was stirred for 1 h. Solvent was removed under vacuum; residue chromatographed to get 5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy) ethyl]-5-methyluridine as white foam (1.95 g, 78%).

5        **5'-O-*tert*-Butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine**  
5'-O-*tert*-butyldiphenylsilyl-2'-O-[(2-formadoximinooxy)ethyl]-5-methyluridine (1.77g, 3.12mmol) was dissolved in a solution of 1M pyridinium p-toluenesulfonate (PPTS) in dry MeOH (30.6mL). Sodium cyanoborohydride (0.39g, 6.13mmol) was added to this solution at 10°C under inert atmosphere. The reaction mixture was stirred for 10 minutes at 10°C. After that the reaction vessel was removed from the ice bath and stirred at room temperature for 2 h, the reaction monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Aqueous NaHCO<sub>3</sub> solution (5%, 10mL) was added and extracted with ethyl acetate (2x20mL). Ethyl acetate phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, evaporated to dryness. Residue was dissolved in a solution of 1M PPTS in MeOH (30.6mL). Formaldehyde (20% w/w, 30mL, 3.37mmol) was added and the reaction mixture was stirred at room temperature for 10 minutes. Reaction mixture cooled to 10°C in an ice bath, sodium cyanoborohydride (0.39g, 6.13mmol) was added and reaction mixture stirred at 10°C for 10 minutes. After 10 minutes, the reaction mixture was removed from the ice bath and stirred at room temperature for 2 hrs. To the reaction mixture 5% NaHCO<sub>3</sub> (25mL) solution was added and extracted with ethyl acetate (2x25mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue obtained was purified by flash column chromatography and eluted with 5% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 5'-O-

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*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine as a white foam (14.6g, 80%).

**2'-O-(dimethylaminoxyethyl)-5-methyluridine**

Triethylamine trihydrofluoride (3.91mL, 24.0mmol) was dissolved in dry THF and triethylamine (1.67mL, 12mmol, dry, kept over KOH). This mixture of triethylamine-2HF was then added to 5'-O-*tert*-butyldiphenylsilyl-2'-O-[N,N-dimethylaminoxyethyl]-5-methyluridine (1.40g, 2.4mmol) and stirred at room temperature for 24 hrs. Reaction was monitored by TLC (5% MeOH in CH<sub>2</sub>Cl<sub>2</sub>). Solvent was removed under vacuum and the residue placed on a flash column and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> to get 2'-O-(dimethylaminoxyethyl)-5-methyluridine (766mg, 92.5%).

**5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine**

2'-O-(dimethylaminoxyethyl)-5-methyluridine (750mg, 2.17mmol) was dried over P<sub>2</sub>O<sub>5</sub> under high vacuum overnight at 40°C. It was then co-evaporated with anhydrous pyridine (20mL). The residue obtained was dissolved in pyridine (11mL) under argon atmosphere. 4-dimethylaminopyridine (26.5mg, 2.60mmol), 4,4'-dimethoxytrityl chloride (880mg, 2.60mmol) was added to the mixture and the reaction mixture was stirred at room temperature until all of the starting material disappeared. Pyridine was removed under vacuum and the residue chromatographed and eluted with 10% MeOH in CH<sub>2</sub>Cl<sub>2</sub> (containing a few drops of pyridine) to get 5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine (1.13g, 80%).

**5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]**

5'-O-DMT-2'-O-(dimethylaminoxyethyl)-5-methyluridine (1.08g, 1.67mmol) was co-evaporated with toluene (20mL). To the residue N,N-diisopropylamine tetrazonide (0.29g, 1.67mmol) was added and dried over P<sub>2</sub>O<sub>5</sub> under high vacuum

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overnight at 40°C. Then the reaction mixture was dissolved in anhydrous acetonitrile (8.4mL) and 2-cyanoethyl-N,N,N<sup>1</sup>,N<sup>1</sup>-tetraisopropylphosphoramidite (2.12mL, 6.08mmol) was added. The reaction mixture was stirred at ambient temperature for 4 hrs under inert atmosphere. The progress of the reaction was monitored by TLC (hexane:ethyl acetate 1:1). The solvent was evaporated, then the residue was dissolved in ethyl acetate (70mL) and washed with 5% aqueous NaHCO<sub>3</sub> (40mL). Ethyl acetate layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated. Residue obtained was chromatographed (ethyl acetate as eluent) to get 5'-O-DMT-2'-O-(2-N,N-dimethylaminoxyethyl)-5-methyluridine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite] as a foam (1.04g, 74.9%).

**2'-(Aminooxyethoxy) nucleoside amidites**

2'-(Aminooxyethoxy) nucleoside amidites [also known in the art as 2'-O-(aminooxyethyl) nucleoside amidites] are prepared as described in the following paragraphs. Adenosine, cytidine and thymidine nucleoside amidites are prepared similarly.

**N2-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite]**

The 2'-O-aminooxyethyl guanosine analog may be obtained by selective 2'-O-alkylation of diaminopurine riboside. Multigram quantities of diaminopurine riboside may be purchased from Schering AG (Berlin) to provide 2'-O-(2-ethylacetyl) diaminopurine riboside along with a minor amount of the 3'-O-isomer. 2'-O-(2-ethylacetyl) diaminopurine riboside may be resolved and converted to 2'-O-(2-ethylacetyl)guanosine by treatment with adenosine deaminase. (McGee, D. P. C., Cook, P. D., Guinosso, C. J., WO 94/02501 A1 940203.) Standard protection procedures

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should afford 2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine and 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-ethylacetyl)-5'-O-(4,4'-dimethoxytrityl)guanosine which may be reduced to provide 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-(2-hydroxyethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine. As before the hydroxyl group may be displaced by N-hydroxyphthalimide via a Mitsunobu reaction, and the protected nucleoside may phosphitylated as usual to yield 2-N-isobutyryl-6-O-diphenylcarbamoyl-2'-O-([2-phthalimidoxy]ethyl)-5'-O-(4,4'-dimethoxytrityl)guanosine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphoramidite].

**2'-dimethylaminoethoxyethoxy (2'-DMAEOE) nucleoside amidites**

2'-dimethylaminoethoxyethoxy nucleoside amidites (also known in the art as 2'-O-dimethylaminoethoxyethyl, i.e., 2'-O-CH<sub>2</sub>-O-CH<sub>2</sub>-N(CH<sub>2</sub>)<sub>2</sub>, or 2'-DMAEOE nucleoside amidites) are prepared as follows. Other nucleoside amidites are prepared similarly.

**2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]-5-methyl uridine**

2[2-(Dimethylamino)ethoxy]ethanol (Aldrich, 6.66 g, 50 mmol) is slowly added to a solution of borane in tetrahydrofuran (1 M, 10 mL, 10 mmol) with stirring in a 100 mL bomb. Hydrogen gas evolves as the solid dissolves. O<sup>2</sup>-,2'-anhydro-5-methyluridine (1.2 g, 5 mmol), and sodium bicarbonate (2.5 mg) are added and the bomb is sealed, placed in an oil bath and heated to 155°C for 26 hours. The bomb is cooled to room temperature and opened. The crude solution is concentrated and the residue partitioned between water (200 mL) and hexanes (200 mL). The excess phenol is extracted into the hexane layer. The aqueous layer is extracted with ethyl acetate (3x200 mL) and the combined organic layers are washed once with water, dried over anhydrous sodium sulfate and concentrated. The residue is



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columned on silica gel using methanol/methylene chloride 1:20 (which has 2% triethylamine) as the eluent. As the column fractions are concentrated a colorless solid forms which is collected to give the title compound as a white solid.

**5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine**

To 0.5 g (1.3 mmol) of 2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine in anhydrous pyridine (8 mL), triethylamine (0.36 mL) and dimethoxytrityl chloride (DMT-Cl, 0.87 g, 2 eq.) are added and stirred for 1 hour. The reaction mixture is poured into water (200 mL) and extracted with CH<sub>2</sub>Cl<sub>2</sub> (2x200 mL). The combined CH<sub>2</sub>Cl<sub>2</sub> layers are washed with saturated NaHCO<sub>3</sub> solution, followed by saturated NaCl solution and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by silica gel chromatography using MeOH:CH<sub>2</sub>Cl<sub>2</sub>:Et<sub>3</sub>N (20:1, v/v, with 1% triethylamine) gives the title compound.

**5'-O-Dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyl uridine-3'-O-(cyanoethyl-N,N-diisopropyl)phosphoramidite**

Diisopropylaminotetrazolide (0.6 g) and 2-cyanoethoxy-N,N-diisopropyl phosphoramidite (1.1 mL, 2 eq.) are added to a solution of 5'-O-dimethoxytrityl-2'-O-[2(2-N,N-dimethylaminoethoxy)ethyl]]-5-methyluridine (2.17 g, 3 mmol) dissolved in CH<sub>2</sub>Cl<sub>2</sub> (20 mL) under an atmosphere of argon. The reaction mixture is stirred overnight and the solvent evaporated. The resulting residue is purified by silica gel flash column chromatography with ethyl acetate as the eluent to give the title compound.

**Example 2**

**Oligonucleotide synthesis**

Unsubstituted and substituted phosphodiester (P=O)

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oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as for the  
5 phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 sec and was followed by the  
10 capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 h), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.

Phosphinate oligonucleotides are prepared as described  
15 in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are  
20 prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent, 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

25 Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are  
30 prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by

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reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

5 **Example 3**

**Oligonucleoside Synthesis**

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethylhydrazo linked oligonucleosides, also identified as  
10 MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligonucleosides, also identified as amide-4 linked oligonucleosides, as well as mixed backbone compounds having, for  
15 instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides  
20 are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

25 **Example 4**

**PNA Synthesis**

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential  
30 Applications, *Bioorganic & Medicinal Chemistry*, 1996, 4, 5-23. They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

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**Example 5****Synthesis of Chimeric Oligonucleotides**

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

**[2'-O-Me] -- [2'-deoxy] -- [2'-O-Me] Chimeric  
Phosphorothioate Oligonucleotides**

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 ammonia/ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hrs at room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hrs at room

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temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

**[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides**

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

**[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides**

[2'-O-(2-methoxyethyl phosphodiester)]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,4-dithiolane-2-one 1,1 dioxides (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to United States patent 5,623,065, herein incorporated by reference.

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**Example 6****Oligonucleotide Isolation**

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by <sup>31</sup>P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

**Example 7****Oligonucleotide Synthesis - 96 Well Plate Format**

Oligonucleotides were synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages were afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages were generated by sulfurization utilizing 3,4-dithiolane-2-thione 1,1-dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl

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phosphoramidites.

Oligonucleotides were cleaved from support and deprotected with concentrated  $\text{NH}_4\text{OH}$  at elevated temperature (55-60°C) for 12-16 hours and the released product then  
5 dried in vacuo. The dried product was then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

#### **Example 8**

##### **10 Oligonucleotide Analysis - 96 Well Plate Format**

The concentration of oligonucleotide in each well was assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products was evaluated by capillary electrophoresis (CE) in  
15 either the 96 well format (Beckman P/ACE™ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACE™ 5000, ABI 270). Base and backbone composition was confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test  
20 plates were diluted from the master plate using single and multi-channel robotic pipettors. Plates were judged to be acceptable if at least 85% of the compounds on the plate were at least 85% full length.

#### **Example 9**

##### **25 Cell culture and oligonucleotide treatment**

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable levels. This can be routinely determined using,  
30 for example, PCR or Northern blot analysis. The following 5 cell types are provided for illustrative purposes, but other cell types can be routinely used, provided that the target is expressed in the cell type chosen. This can be readily

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determined by methods routine in the art, for example Northern blot analysis, Ribonuclease protection assays, or RT-PCR.

T-24 cells:

5       The human transitional cell bladder carcinoma cell line T-24 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells were routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum  
10 (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well  
15 plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium  
20 and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells were routinely cultured in DMEM basal media  
25 (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by  
30 trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) were obtained



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from the Clonetics Corporation (Walkersville MD). NHDFs were routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells were maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) were obtained from the Clonetics Corporation (Walkersville MD). HEKs were routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells were routinely maintained for up to 10 passages as recommended by the supplier.

b.END cells:

The mouse brain endothelial cell line b.END was obtained from Dr. Werner Risau at the Max Plank Institute (Bad Nauheim, Germany). b.END cells were routinely cultured in DMEM, high glucose (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD). Cells were routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells were seeded into 96-well plates (Falcon-Primaria #3872) at a density of 3000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analyses, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

Treatment with antisense compounds:

When cells reached 80% confluency, they were treated with oligonucleotide. For cells grown in 96-well plates, wells were washed once with 200  $\mu$ L OPTI-MEM™-1 reduced-serum medium (Gibco BRL) and then treated with 130  $\mu$ L of OPTI-

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MEM™-1 containing 3.75 µg/mL LIPOFECTIN™ (Gibco BRL) and the desired concentration of oligonucleotide. After 4-7 hours of treatment, the medium was replaced with fresh medium. Cells were harvested 16-24 hours after  
5 oligonucleotide treatment.

The concentration of oligonucleotide used varies from cell line to cell line. To determine the optimal oligonucleotide concentration for a particular cell line, the cells are treated with a positive control  
10 oligonucleotide at a range of concentrations. For human cells the positive control oligonucleotide is ISIS 13920, **TCCGTCATCGCTCCTCAGGG**, SEQ ID NO: 1, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to human H-ras.  
15 For mouse or rat cells the positive control oligonucleotide is ISIS 15770, **ATGCATTCTGCCCCAAGGA**, SEQ ID NO: 2, a 2'-O-methoxyethyl gapmer (2'-O-methoxyethyls shown in bold) with a phosphorothioate backbone which is targeted to both mouse and rat c-raf. The concentration of positive control  
20 oligonucleotide that results in 80% inhibition of c-Ha-ras (for ISIS 13920) or c-raf (for ISIS 15770) mRNA is then utilized as the screening concentration for new oligonucleotides in subsequent experiments for that cell line. If 80% inhibition is not achieved, the lowest  
25 concentration of positive control oligonucleotide that results in 60% inhibition of H-ras or c-raf mRNA is then utilized as the oligonucleotide screening concentration in subsequent experiments for that cell line. If 60% inhibition is not achieved, that particular cell line is  
30 deemed as unsuitable for oligonucleotide transfection experiments.

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**Example 10****Analysis of oligonucleotide inhibition of Casein kinase 2-beta expression**

Antisense modulation of Casein kinase 2-beta expression  
5 can be assayed in a variety of ways known in the art. For  
example, Casein kinase 2-beta mRNA levels can be quantitated  
by, e.g., Northern blot analysis, competitive polymerase  
chain reaction (PCR), or real-time PCR (RT-PCR). Real-time  
quantitative PCR is presently preferred. RNA analysis can  
10 be performed on total cellular RNA or poly(A)+ mRNA.  
Methods of RNA isolation are taught in, for example,  
Ausubel, F.M. et al., *Current Protocols in Molecular  
Biology*, Volume 1, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3, John  
Wiley & Sons, Inc., 1993. Northern blot analysis is routine  
15 in the art and is taught in, for example, Ausubel, F.M. et  
al., *Current Protocols in Molecular Biology*, Volume 1, pp.  
4.2.1-4.2.9, John Wiley & Sons, Inc., 1996. Real-time  
quantitative (PCR) can be conveniently accomplished using  
the commercially available ABI PRISM™ 7700 Sequence  
20 Detection System, available from PE-Applied Biosystems,  
Foster City, CA and used according to manufacturer's  
instructions.

Protein levels of Casein kinase 2-beta can be  
quantitated in a variety of ways well known in the art, such  
25 as immunoprecipitation, Western blot analysis  
(immunoblotting), ELISA or fluorescence-activated cell  
sorting (FACS). Antibodies directed to Casein kinase 2-beta  
can be identified and obtained from a variety of sources,  
such as the MSRS catalog of antibodies (Aerie Corporation,  
30 Birmingham, MI), or can be prepared via conventional  
antibody generation methods. Methods for preparation of  
polyclonal antisera are taught in, for example, Ausubel,  
F.M. et al., *Current Protocols in Molecular Biology*, Volume

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2, pp. 11.12.1-11.12.9, John Wiley & Sons, Inc., 1997. Preparation of monoclonal antibodies is taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.4.1-11.11.5, John Wiley & Sons, Inc., 1997.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.16.1-10.16.11, John Wiley & Sons, Inc., 1998. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 10.8.1-10.8.21, John Wiley & Sons, Inc., 1997. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 2, pp. 11.2.1-11.2.22, John Wiley & Sons, Inc., 1991.

#### Example 11

##### Poly(A)+ mRNA isolation

Poly(A)+ mRNA was isolated according to Miura et al., *Clin. Chem.*, 1996, 42, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, F.M. et al., *Current Protocols in Molecular Biology*, Volume 1, pp. 4.5.1-4.5.3, John Wiley & Sons, Inc., 1993. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200  $\mu$ L cold PBS. 60  $\mu$ L lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) was added to each well, the plate was gently agitated and then incubated at room temperature for five minutes. 55  $\mu$ L of lysate was transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates were incubated for 60 minutes at room temperature, washed 3 times

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with 200  $\mu$ L of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate was blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60  $\mu$ L of elution buffer (5 mM  
5 Tris-HCl pH 7.6), preheated to 70°C was added to each well, the plate was incubated on a 90°C hot plate for 5 minutes, and the eluate was then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be  
10 treated similarly, using appropriate volumes of all solutions.

**Example 12****Total RNA Isolation**

Total RNA was isolated using an RNEASY 96™ kit and  
15 buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium was removed from the cells and each well was washed with 200  $\mu$ L cold PBS. 100  $\mu$ L Buffer RLT was added to each well and the plate  
20 vigorously agitated for 20 seconds. 100  $\mu$ L of 70% ethanol was then added to each well and the contents mixed by pipetting three times up and down. The samples were then transferred to the RNEASY 96™ well plate attached to a QIAVAC™ manifold fitted with a waste collection tray and  
25 attached to a vacuum source. Vacuum was applied for 15 seconds. 1 mL of Buffer RW1 was added to each well of the RNEASY 96™ plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE was then added to each well of the RNEASY 96™ plate and the vacuum applied for a period of  
30 15 seconds. The Buffer RPE wash was then repeated and the vacuum was applied for an additional 10 minutes. The plate

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was then removed from the QIAVAC™ manifold and blotted dry on paper towels. The plate was then re-attached to the QIAVAC™ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA was then eluted by  
5 pipetting 60 µL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step was repeated with an additional 60 µL water.

The repetitive pipetting and elution steps may be automated using a QIAGEN Bio-Robot 9604 (Qiagen, Inc.,  
10 Valencia CA). Essentially, after lysing of the cells on the culture plate, the plate is transferred to the robot deck where the pipetting, DNase treatment and elution steps are carried out.

### **Example 13**

#### **15 Real-time Quantitative PCR Analysis of Casein kinase 2-beta mRNA Levels**

Quantitation of Casein kinase 2-beta mRNA levels was determined by real-time quantitative PCR using the ABI PRISM™ 7700 Sequence Detection System (PE-Applied  
20 Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which  
25 amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR  
30 primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE, FAM, or VIC, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems,

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Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe.

5 When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-  
exonuclease activity of Taq polymerase. During the  
10 extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye  
15 molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISM™ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated  
20 control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

Prior to quantitative PCR analysis, primer-probe sets specific to the target gene being measured are evaluated for  
25 their ability to be "multiplexed" with a GAPDH amplification reaction. In multiplexing, both the target gene and the internal standard gene GAPDH are amplified concurrently in a single sample. In this analysis, mRNA isolated from untreated cells is serially diluted. Each dilution is  
30 amplified in the presence of primer-probe sets specific for GAPDH only, target gene only ("single-plexing"), or both (multiplexing). Following PCR amplification, standard curves of GAPDH and target mRNA signal as a function of

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dilution are generated from both the single-plexed and multiplexed samples. If both the slope and correlation coefficient of the GAPDH and target signals generated from the multiplexed samples fall within 10% of their

5 corresponding values generated from the single-plexed samples, the primer-probe set specific for that target is deemed multiplexable. Other methods of PCR are also known in the art.

PCR reagents were obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions were carried out by adding 25  $\mu$ L PCR cocktail (1x TAQMAN<sup>TM</sup> buffer A, 5.5 mM MgCl<sub>2</sub>, 300  $\mu$ M each of dATP, dCTP and dGTP, 600  $\mu$ M of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNase inhibitor, 1.25 Units AMPLITAQ GOLD<sup>TM</sup>, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25  $\mu$ L total RNA solution. The RT reaction was carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLD<sup>TM</sup>, 40 cycles of a two-step PCR protocol were carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Gene target quantities obtained by real time RT-PCR are normalized using either the expression level of GAPDH, a gene whose expression is constant, or by quantifying total RNA using RiboGreen<sup>TM</sup> (Molecular Probes, Inc. Eugene, OR). GAPDH expression is quantified by real time RT-PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RiboGreen<sup>TM</sup> RNA quantification reagent from Molecular Probes. Methods of RNA quantification by RiboGreen<sup>TM</sup> are taught in Jones, L.J., et al, *Analytical Biochemistry*, 1998, 265, 368-374.



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In this assay, 175  $\mu$ L of RiboGreen<sup>TM</sup> working reagent (RiboGreen<sup>TM</sup> reagent diluted 1:2865 in 10mM Tris-HCl, 1 mM EDTA, pH 7.5) is pipetted into a 96-well plate containing 25uL purified, cellular RNA. The plate is read in a  
5 CytoFluor 4000 (PE Applied Biosystems) with excitation at 480nm and emission at 520nm.

Probes and primers to human Casein kinase 2-beta were designed to hybridize to a human Casein kinase 2-beta sequence, using published sequence information (GenBank  
10 accession number 1593\_041C, incorporated herein as SEQ ID NO:3). For human Casein kinase 2-beta the PCR primers were: forward primer: CCCTCACATGCTCTTCATGGT (SEQ ID NO: 4) reverse primer: GCCTGGGCACAACTGGTT (SEQ ID NO: 5) and the PCR probe was: FAM-CATCCCGAGTACCGGCCCAAGAG-TAMRA  
15 (SEQ ID NO: 6) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For human GAPDH the PCR primers were: forward primer: GAAGGTGAAGGTCGGAGTC (SEQ ID NO: 7)  
20 reverse primer: GAAGATGGTGATGGGATTTTC (SEQ ID NO: 8) and the PCR probe was: 5' JOE-CAAGCTTCCCGTTCTCAGCCX- TAMRA 3' (SEQ ID NO: 9) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye.

25 Probes and primers to mouse Casein kinase 2-beta were designed to hybridize to a mouse Casein kinase 2-beta sequence, using published sequence information (GenBank accession number 1593\_041D, incorporated herein as SEQ ID NO:10). For mouse Casein kinase 2-beta the PCR primers  
30 were: forward primer: GATCCACGCCCCGCTACAT (SEQ ID NO:11) reverse primer: TCCCTGCTGGTACTTTTCCAA (SEQ ID NO: 12) and the PCR probe was: FAM-CACCAACCGAGGCATCGCACAA-TAMRA

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(SEQ ID NO: 13) where FAM (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-Applied Biosystems, Foster City, CA) is the quencher dye. For mouse GAPDH the PCR primers were:

5 forward primer: GGCAAATTCAACGGCACAGT (SEQ ID NO: 14)  
reverse primer: GGGTCTCGCTCCTGGAAGAT (SEQ ID NO: 15) and the  
PCR probe was: 5' JOE-AAGGCCGAGAATGGGAAGCTTGTGTCATCX- TAMRA 3'  
(SEQ ID NO: 16) where JOE (PE-Applied Biosystems, Foster City, CA) is the fluorescent reporter dye) and TAMRA (PE-  
10 Applied Biosystems, Foster City, CA) is the quencher dye.

#### **Example 14**

##### **Northern blot analysis of Casein kinase 2-beta mRNA levels**

Eighteen hours after antisense treatment, cell monolayers were washed twice with cold PBS and lysed in 1 mL  
15 RNAZOL™ (TEL-TEST "B" Inc., Friendswood, TX). Total RNA was prepared following manufacturer's recommended protocols. Twenty micrograms of total RNA was fractionated by electrophoresis through 1.2% agarose gels containing 1.1% formaldehyde using a MOPS buffer system (AMRESCO, Inc.  
20 Solon, OH). RNA was transferred from the gel to HYBOND™-N+ nylon membranes (Amersham Pharmacia Biotech, Piscataway, NJ) by overnight capillary transfer using a Northern/Southern Transfer buffer system (TEL-TEST "B" Inc., Friendswood, TX). RNA transfer was confirmed by UV visualization. Membranes  
25 were fixed by UV cross-linking using a STRATALINKER™ UV Crosslinker 2400 (Stratagene, Inc, La Jolla, CA) and then probed using QUICKHYB™ hybridization solution (Stratagene, La Jolla, CA) using manufacturer's recommendations for stringent conditions.

30 To detect human Casein kinase 2-beta, a human Casein kinase 2-beta specific probe was prepared by PCR using the forward primer CCCTCACATGCTCTTCATGGT (SEQ ID NO: 4) and the

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reverse primer GCCTGGGCACAACTGGTT (SEQ ID NO: 5). To  
normalize for variations in loading and transfer efficiency  
membranes were stripped and probed for human glyceraldehyde-  
3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto,  
5 CA).

To detect mouse Casein kinase 2-beta, a mouse Casein  
kinase 2-beta specific probe was prepared by PCR using the  
forward primer GATCCACGCCCGCTACAT (SEQ ID NO:11) and the  
reverse primer TCCCTGCTGGTACTTTTCCAA (SEQ ID NO: 12). To  
10 normalize for variations in loading and transfer efficiency  
membranes were stripped and probed for mouse glyceraldehyde-  
3-phosphate dehydrogenase (GAPDH) RNA (Clontech, Palo Alto,  
CA).

Hybridized membranes were visualized and quantitated  
15 using a PHOSPHORIMAGER™ and IMAGEQUANT™ Software V3.3  
(Molecular Dynamics, Sunnyvale, CA). Data was normalized to  
GAPDH levels in untreated controls.

#### **Example 15**

**Antisense inhibition of human Casein kinase 2-beta  
20 expression by chimeric phosphorothioate oligonucleotides  
having 2'-MOE wings and a deoxy gap**

In accordance with the present invention, a series of  
oligonucleotides were designed to target different regions  
of the human Casein kinase 2-beta RNA, using published  
25 sequences (mRNA of human casein kinase 2-beta extracted from  
GenBank accession number X57152, incorporated herein as SEQ  
ID NO: 3, and GenBank accession number X57152, incorporated  
herein as SEQ ID NO: 17). The oligonucleotides are shown in  
Table 1. "Target site" indicates the first (5'-most)  
30 nucleotide number on the particular target sequence to which  
the oligonucleotide binds. All compounds in Table 1 are  
chimeric oligonucleotides ("gapmers") 20 nucleotides in  
length, composed of a central "gap" region consisting of ten

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2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. The compounds were analyzed for their effect on human Casein kinase 2-beta mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 1

Inhibition of human Casein kinase 2-beta mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
126985	5'UTR	17	1039	cgctcccagcgacaagggac	29	18
126986	5'UTR	17	1059	agtccggagaccccatgtgc	35	19
126987	5'UTR	17	1338	ctcccgaaggccagggccct	34	20
126988	5'UTR	17	1374	tactttgacccaccagcccg	18	21
126989	Coding	17	1622	ctgctcatcttcacgtcagc	91	22
126990	Intron	17	1875	agactcaggacttgggccc	60	23
126991	Intron	17	2000	agaggtaggcagtaagccgag	60	24
126992	Intron	17	2017	gcttgaacctgggaggcaga	42	25
126993	Intron	17	2076	caggcatggtggcaggcact	0	26
126994	Intron	17	2293	caatgtgtagcatttcagtt	63	27
126995	Intron	17	2350	tctcagctggctaaggaagg	50	28
126996	Intron	17	2374	cgtctctaggaaccagtttt	56	29
126997	Intron	17	2588	tgctctaagtgatgccttat	65	30
126998	Intron	17	3033	ctggaaggtgcaaagcagga	45	31
126999	Intron	17	3192	gcattgggacccagaaacctg	65	32
127000	Intron	17	3241	tcagtttgcttagcacctac	61	33
127001	Intron	17	3245	ctattcagtttgcttagcac	60	34
127002	Intron	17	3250	gcgggctattcagtttgct	69	35
127003	Coding	17	3341	gtcttccagttcttcacctg	30	36
127004	Intron	17	4522	ctcagagctaaagcctcgtg	73	37
127005	5'UTR	3	31	gtcacgaaggcccgaggag	0	38
127006	5'UTR	3	53	atgacccgcgcacgacctgg	0	39
127007	5'UTR	3	95	agaaactggctaaatgagag	6	40
127008	5'UTR	3	105	cggtagagaaagaaactggc	2	41
127009	5'UTR	3	124	tgccgggacacggagtcccc	18	42
127010	5'UTR	3	153	aagcgccaaggggtcaggtgc	25	43
127011	5'UTR	3	167	agagggcaacacgcaagcgc	24	44
127012	5'UTR	3	224	acccgaccgcggcaggcgaa	85	45
127013	5'UTR	3	239	cagcgcaggccgcggacctg	73	46
127014	5'UTR	3	252	gcggcgaccgctacagcgca	74	47

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127015	5'UTR	3	257	gaacggcgggcgaccgctaca	74	48
127016	5'UTR	3	267	tacttccagggaacggcgggc	88	49
127017	Intron: Exon Junction	3	332	gagctgctcatcttcacgtc	71	50
127018	Coding	3	348	ccaggacacctcctctgagc	88	51
127019	Coding	3	353	gaaatccaggacacctcctc	76	52
127020	Coding	3	363	acagaaccaggaaatccagg	87	53
127021	Coding	3	367	gcccacagaaccaggaaatc	70	54
127022	Coding	3	373	cacggagcccacagaaccag	89	55
127023	Coding	3	380	tcattgccacggagcccaca	85	56
127024	Coding	3	394	cttcacagaagaattcattg	72	57
127025	Coding	3	409	tgtagtcttcatccacttca	82	58
127026	Intron: Exon Junction	3	413	tggatgtagtcttcatccac	61	59
127027	Coding	3	423	aaatttgtcctggatgtagt	68	60
127028	Coding	3	433	cagtaagattaaatttgtcc	78	61
127029	Coding	3	443	tcattgagtcacagtaagatt	74	62
127030	Coding	3	470	agagcttgtcgatagtggagg	79	63
127031	Coding	3	473	tctagagcttgtcgatagtg	62	64
127032	Coding	3	484	ccaagatcatgtctagagct	78	65
127033	Coding	3	504	ttcttcatcagggtccaggt	70	66
127034	Coding	3	541	gctcaatcagggtcactctgg	78	67
127035	Coding	3	549	ggctgcctgctcaatcaggt	64	68
127036	Coding	3	578	cgggctggtgatcaatccata	82	69
127037	Coding	3	584	atgtagcgggcggtggatcaa	56	70
127038	Coding	3	629	tgctgggtacttttccaacat	79	71
127039	Coding	3	661	agtacacacgaggacagtaa	74	72
127040	Coding	3	674	ggctgggttctcacagtacac	86	73
127041	Coding	3	677	attggctgggttctcacagta	76	74
127042	Intron: Exon Junction	3	707	cctgggatgtctgaaaggcc	70	75
127043	Coding	3	711	ttcacctgggatgtctgaaa	81	76
127044	Coding	3	761	ttgggtgtgtacacatccat	62	77
127045	Coding	3	774	gtgtcttgatgacttgggtg	45	78
127046	Coding	3	791	gcgccatccgtgtgatgggtg	79	79
127047	Coding	3	804	agtgccgaagttaggcgccat	73	80
127048	Coding	3	812	gggaaaccagtgccgaagta	78	81
127049	Coding	3	822	gagcatgtgagggaaaccag	85	82
127050	Coding	3	832	gcaccatgaagagcatgtga	92	83
127051	Coding	3	860	gcaggctctcttgggcccgtg	87	84
127052	Coding	3	886	cgtagagcctgggcacaaac	69	85
127053	Coding	3	930	ggcttgagctgcagctgggt	57	86
127054	Coding	3	940	agttgctggcggttgaggc	68	87
127055	Coding	3	950	gggctcttgaagttgctggc	65	88
127056	Coding	3	961	tcgtcttgactgggctcttg	80	89
127057	Coding	3	977	ggagggaatcagcgaatcgt	66	90
127058	3' UTR	3	1035	ggttcctgaaagggtggcaa	82	91
127059	3' UTR	3	1064	tcctttaatttaactaaaa	19	92
127060	3' UTR	3	1077	ccacgataacgactccttta	79	93
127061	3' UTR	3	1093	ttatttcatattcccaccac	57	94
127062	3' UTR	3	1101	cttctactttatttcatatt	74	95

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As shown in Table 1, SEQ ID NOs 22, 23, 24, 25, 27, 28, 29, 30, 31, 32, 33, 34, 35, 37, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 93, 94 and 95 demonstrated at least 40% inhibition of human Casein kinase 2-beta expression in this assay and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of the present invention.

**Example 16**

**Antisense inhibition of mouse Casein kinase 2-beta expression by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap.**

In accordance with the present invention, a second series of oligonucleotides were designed to target different regions of the mouse Casein kinase 2-beta RNA, using published sequences (mRNA of the mouse casein kinase 2-beta gene extracted from GenBank accession number X80685, incorporated herein as SEQ ID NO: 10, and GenBank accession number X80685, incorporated herein as SEQ ID NO: 96). The oligonucleotides are shown in Table 2. "Target site" indicates the first (5'-most) nucleotide number on the particular target sequence to which the oligonucleotide binds. All compounds in Table 2 are chimeric oligonucleotides ("gapmers") 20 nucleotides in length, composed of a central "gap" region consisting of ten 2'-deoxynucleotides, which is flanked on both sides (5' and 3' directions) by five-nucleotide "wings". The wings are composed of 2'-methoxyethyl (2'-MOE)nucleotides. The internucleoside (backbone) linkages are phosphorothioate (P=S) throughout the oligonucleotide. All cytidine residues

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are 5-methylcytidines. The compounds were analyzed for their effect on mouse Casein kinase 2-beta mRNA levels by quantitative real-time PCR as described in other examples herein. Data are averages from two experiments. If present, "N.D." indicates "no data".

Table 2

Inhibition of mouse Casein kinase 2-beta mRNA levels by chimeric phosphorothioate oligonucleotides having 2'-MOE wings and a deoxy gap

ISIS #	REGION	TARGET SEQ ID NO	TARGET SITE	SEQUENCE	%INHIB	SEQ ID NO
127019	Coding	10	345	gaaatccaggacacctcctc	24	52
127020	Coding	10	355	acagaaccaggaaatccagg	77	53
127022	Coding	10	365	cacggagcccacagaaccag	87	55
127026	Intron: Exon Junction	10	405	tggatgtagtcttcatccac	67	59
127027	Coding	10	415	aaatttgctcctggatgtagt	23	60
127028	Coding	10	425	cagtaagattaaatttgctcc	74	61
127029	Coding	10	435	tcattgagtcacagtaagatt	29	62
127030	Coding	10	462	agagcttgctcgatagtgagg	77	63
127037	Coding	10	576	atgtagcgggctggatcaa	83	70
127038	Coding	10	621	tgctgggtacttttccaacat	76	71
127041	Coding	10	669	attggctgggtctcacagta	64	74
127042	Intron: Exon Junction	10	699	cctgggatgtctgaaaggcc	79	75
127048	Coding	10	804	gggaaaccagtgccgaagta	80	81
127049	Coding	10	814	gagcatgtgagggaaccag	77	82
127050	Coding	10	824	gcaccatgaagagcatgtga	87	83
127053	Coding	10	922	ggcttggagctgcagctggt	78	86
127054	Coding	10	932	agttgctggcggcttgaggc	72	87
127055	Coding	10	942	gggctcttgaagttgctggc	63	88
127059	3'UTR	10	1054	tcctttaatttaaactaaaa	54	92
127061	3'UTR	10	1083	ttatttcatattcccaccac	86	94
127063	5'UTR	96	1734	ttggcgaaggcaccacactt	54	97
127064	5'UTR	96	1944	tccagaaaatgacaggctcct	71	98
127065	Coding	96	2237	gccgacctaaactcacctca	59	99
127066	Intron	96	2290	cgaaatctgctctcaaacaa	76	100
127067	Intron	96	2389	tgcatattgacaaaaccttg	46	101
127068	Intron	96	2605	cgacacaagcttatttttaga	75	102
127069	Intron	96	2709	tgctctattcccaattctgc	67	103
127070	Coding	96	2821	gtcttcatccacctacgggc	64	104
127071	Intron	96	3157	taaattcaattcccagcaac	54	105
127072	Intron	96	3173	tcgagagcagagttcctaaa	84	106
127073	Intron	96	3336	tcctgagttcaaattcccagc	77	107
127074	Intron	96	3423	ctagaacagacaaggctacc	72	108
127075	Intron	96	3616	gttatgaatgcttgggcctt	73	109
127076	Intron	96	3833	aggccctctagagcagcaga	51	110

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127077	Intron	96	4013	tctctagtagctgtttctga	81	111
127078	Intron	96	5566	ggatgggttggcaggcatga	44	112
127079	Intron	96	5831	tgctcttccaaaggtcggaa	35	113
127080	Intron	96	5994	aaccatccccagcttaaaaa	82	114
127081	Intron	96	6008	gcctccccttgtgttaacct	66	115
127082	Intron	96	6164	cctcacatcgactccacagg	69	116
127083	5' UTR	10	45	accaatcccaagaggaccgcg	1	117
127084	5' UTR	10	58	gggaagacaaaactaccaatc	0	118
127085	5' UTR	10	84	cggcagagagaaaactgacga	12	119
127086	5' UTR	10	98	agatgtagtcccgtcggcag	0	120
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127105	Coding	10	630	aagtctccctgctgggtactt	83	139
127106	Coding	10	656	cacagtatacacaggagacag	87	140
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127112	Coding	10	903	taagccattggatggatctt	75	146
127113	Coding	10	912	tgcagctggtaagccattgg	40	147
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127118	3' UTR	10	1002	gaatgggtggtgtcacagaca	85	152
127119	3' UTR	10	1062	gtaacaattcctttaattta	79	153
127120	3' UTR	10	1090	tcttcctttatttcatatc	83	154

As shown in Table 2, SEQ ID NOS 53, 55, 59, 61, 63, 70, 71, 74, 75, 81, 82, 83, 86, 87, 88, 92, 94, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 114, 115, 116, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153 and 154 demonstrated at least 40% inhibition of mouse Casein kinase 2-beta



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expression in this experiment and are therefore preferred. The target sites to which these preferred sequences are complementary are herein referred to as "active sites" and are therefore preferred sites for targeting by compounds of  
5 the present invention.

**Example 17****Western blot analysis of Casein kinase 2-beta protein levels**

Western blot analysis (immunoblot analysis) is carried out using standard methods. Cells are harvested 16-20 h  
10 after oligonucleotide treatment, washed once with PBS, suspended in Laemmli buffer (100 ul/well), boiled for 5 minutes and loaded on a 16% SDS-PAGE gel. Gels are run for 1.5 hours at 150 V, and transferred to membrane for western blotting. Appropriate primary antibody directed to Casein  
15 kinase 2-beta is used, with a radiolabelled or fluorescently labeled secondary antibody directed against the primary antibody species. Bands are visualized using a PHOSPHORIMAGER™ (Molecular Dynamics, Sunnyvale CA).

**What is claimed is:**

1. A compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding Casein kinase 2-beta, wherein said compound specifically hybridizes with and inhibits the expression of Casein kinase 2-beta.

2. The compound of claim 1 which is an antisense oligonucleotide.

3. The compound of claim 2 wherein the antisense oligonucleotide has a sequence comprising SEQ ID NO: 22, 23, 24, 25, 27, 28, 29, 30, 31, 32, 33, 34, 35, 37, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 93, 94, 95, 92, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 114, 115, 116, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153 or 154.

4. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified internucleoside linkage.

5. The compound of claim 4 wherein the modified internucleoside linkage is a phosphorothioate linkage.

6. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified sugar moiety.

7. The compound of claim 6 wherein the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety.

8. The compound of claim 2 wherein the antisense oligonucleotide comprises at least one modified nucleobase.

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9. The compound of claim 8 wherein the modified nucleobase is a 5-methylcytosine.

10. The compound of claim 2 wherein the antisense oligonucleotide is a chimeric oligonucleotide.

11. A compound 8 to 50 nucleobases in length which specifically hybridizes with at least an 8-nucleobase portion of an active site on a nucleic acid molecule encoding Casein kinase 2-beta.

12. A composition comprising the compound of claim 1 and a pharmaceutically acceptable carrier or diluent.

13. The composition of claim 12 further comprising a colloidal dispersion system.

14. The composition of claim 12 wherein the compound is an antisense oligonucleotide.

15. A method of inhibiting the expression of Casein kinase 2-beta in cells or tissues comprising contacting said cells or tissues with the compound of claim 1 so that expression of Casein kinase 2-beta is inhibited.

16. A method of treating an animal having a disease or condition associated with Casein kinase 2-beta comprising administering to said animal a therapeutically or prophylactically effective amount of the compound of claim 1 so that expression of Casein kinase 2-beta is inhibited.

17. The method of claim 16 wherein the disease or condition is a hyperproliferative disorder.

18. The method of claim 17 wherein the hyperproliferative disorder is cancer.

19. The method of claim 18 wherein the cancer is breast cancer, prostate cancer or liver cancer.

20. The method of claim 16 wherein the disease or condition is diabetes.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE  
Before the United States Receiving Office  
for the Patent Cooperation Treaty

In re application of:                      Isis Pharmaceuticals, Inc. et  
al.

International Filing Date:              31 January 2002

Title:              **Antisense Modulation of Casein Kinase 2-Beta  
Expression**

Agent's file reference:    RTSP-0270

STATEMENT CONCERNING SEQUENCE LISTING

Dear Sirs:

Applicants submit herewith a written form and  
computer readable form of the sequence listing in connection  
with the above International Application. The information  
recorded on the diskette is identical to the written sequence  
listing.

Respectfully submitted,

*Jane Massey Licata*  
Jane Massey Licata  
Registration No. 32,257

Date: 31 January 2002

Licata & Tyrrell P.C.  
66 E. Main Street  
Marlton, New Jersey 08053  
(856) 810-1515

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## SEQUENCE LISTING

<110> Isis Pharmaceuticals, Inc.  
 Robert McKay  
 Susan M. Freier  
 Jacqueline Wyatt

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-4-

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agt acc ggc cca aga gac ctg cca acc agt ttg tgc cca ggt agg gag 4325
Ser Thr Gly Pro Arg Asp Leu Pro Thr Ser Leu Cys Pro Gly Arg Glu
          195                      200                      205

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```

cag gga gag tca tta agg gtc aaa gga aag gcc caa gat ccc cca gag 4373
Gln Gly Glu Ser Leu Arg Val Lys Gly Lys Ala Gln Asp Pro Pro Glu
          210                      215                      220

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```

agg gga gga cag ggc atg gcc ctt tct tga ggtctgcttc tcccagaatc 4423
Arg Gly Gly Gln Gly Met Ala Leu Ser  *
225                      230

```

```

agggcatctc cctgctgagt gactgtggga aagttatttg attatctgtg cttgagttac 4483
cttattgtag aatgttcttg agctgagaag ttgggaacca cgaggcttta gctctgagca 4543
ggcccataga ggagctcagg tggggagggtg ggaatgcagg tgactggcag ggcctggatg 4603
gggctcatgc tgctgcctct ctgacctctg ccctggccta ggctctacgg tttcaagatc 4663
catccg atg gcc tac cag ctg cag ctc caa gcc gcc agc aac ttc aag 4711
      Met Ala Tyr Gln Leu Gln Gln Ala Ala Ser Asn Phe Lys
          235                      240                      245

```

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agc cca gtc aag acg att cgc tga ttcctcccc cacctgtcct gcagtccttg 4765
Ser Pro Val Lys Thr Ile Arg  *
          250

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tcttttcctt tcttttttgc caccctttca ggaaccctgt atggttttta gtttaaatta 4825
aaggagtcgt tatcgtgggtg ggaatatgaa ataaagtaga agaaaaggcc atgagctagt 4885
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ctgttgatg acgaagacag cacatgggtg cagagataga tactaaccga tggactttcc 5425
aaggggagga ataggtcttt ggagggtatg caagacaaag gtagacactg gataaagaac 5485
cgggtagtgc ccagggtatta ccccatctgg gccattactc ccacactcag gaaccagacg 5545

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-9-

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ttgtgggtga ggacatgotg tccctcctgc caagtaataa cttccttccc agccaggatc 5605
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accaccctgc agcttaggat taaggagcat ggtcacagga aggtgggggtt tcagggcatc 5725
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gcccgaggat gg                                     5917

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&lt;210&gt; 18

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 18

cgctcccagc gacaaggac

20

&lt;210&gt; 19

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 19

agtccggaga ccccatgtgc

20

&lt;210&gt; 20

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 20

ctcccgaagg ccagggcct

20

&lt;210&gt; 21

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

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&lt;210&gt; 27

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 27

caatgtgtag catttcagtt

20

&lt;210&gt; 28

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 28

tctcagctgg ctaaggaagg

20

&lt;210&gt; 29

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 29

cgtctctagg aaccagtttt

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&lt;210&gt; 30

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

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&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 36

gtcttccagt tcttcatctg

20

&lt;210&gt; 37

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 37

ctcagagcta aagcctcgtg

20

&lt;210&gt; 38

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 38

gtcacgaagg cccggaggag

20

&lt;210&gt; 39

&lt;211&gt; 20

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&lt;213&gt; Artificial Sequence

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&lt;223&gt; Antisense Oligonucleotide

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&lt;210&gt; 45

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&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 45

acccgaccgc ggcaggcgaa

20

&lt;210&gt; 46

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 46

cagcgcaggc cgcggaccgc

20

&lt;210&gt; 47

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 47

gcggcgaccg ctacagcgca

20

&lt;210&gt; 48

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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gaacggcggc gaccgctaca

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&lt;223&gt; Antisense Oligonucleotide

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acagaaccag gaaatccagg

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&lt;210&gt; 54

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&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 54

gcccacagaa ccaggaaatc

20

&lt;210&gt; 55

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 55

cacggagccc acagaaccag

20

&lt;210&gt; 56

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 56

tcattgccac ggagcccaca

20

&lt;210&gt; 57

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 57

cttcacagaa gaattcattg

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&lt;210&gt; 58

-18-

<211> 20  
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<210> 66  
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<210> 67  
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cctgggatgt ctgaaaggcc 20

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&lt;213&gt; Artificial Sequence

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&lt;223&gt; Antisense Oligonucleotide

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&lt;210&gt; 77

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 77

ttgggtgtgt acacatccat

20

&lt;210&gt; 78

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 78

gtgtcttgat gacttgggtg

20

&lt;210&gt; 79

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 79

gcgccatccg tgtgatggtg

20

&lt;210&gt; 80

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

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&lt;400&gt; 80

-23-

agtgccgaag taggcgccat 20

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&lt;220&gt;

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&lt;400&gt; 85

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&lt;210&gt; 86

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 86

ggcttgagc tgcagctggt

20

&lt;210&gt; 87

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 87

agttgctggc ggcttgagc

20

&lt;210&gt; 88

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 88

gggctcttga agttgctggc

20

&lt;210&gt; 89

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

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-25-

<210> 90  
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&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 94

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20

&lt;210&gt; 95

&lt;211&gt; 20

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Antisense Oligonucleotide

&lt;400&gt; 95

cttctactttt atttcatatt

20

&lt;210&gt; 96

&lt;211&gt; 7874

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (2169) ... (2297)

&lt;221&gt; CDS

&lt;222&gt; (4112) ... (4150)

&lt;221&gt; CDS

&lt;222&gt; (2833) ... (2913)

&lt;221&gt; CDS

&lt;222&gt; (4576) ... (4614)

&lt;221&gt; CDS

&lt;222&gt; (4827) ... (4994)

&lt;221&gt; CDS

&lt;222&gt; (6267) ... (6431)

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-27-

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Met Ser Ser Ser Glu Glu Val Ser Trp Ile Ser Trp Phe Cys
1 5 10

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Gly Leu Arg Gly Asn Glu Phe Phe Cys Glu Val Ser Leu Gly Arg Pro
15 20 25 30

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act cca cat gcc ctc gat gtt tta ttt tat gtt gtt tga gagcagattt 2307
Thr Pro His Ala Leu Asp Val Leu Phe Tyr Val Val *
35 40

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Met Lys Thr Thr
45

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Ser Arg Thr Asn Leu Ile Leu Leu Asp Ser Met Ser Arg Cys Leu Thr
50 55 60

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atc gac aag ctc tgg aca tga tcttagacct ggaacctggg aatgtgccct 2943
Ile Asp Lys Leu Trp Thr *
65

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-28-

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Met Lys Ser Trp Lys Thr Thr Pro
70 75

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Thr Arg Ala Thr *
80

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Met Leu Thr His Gln Ser Leu Leu Phe
85

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tac aca cct tag ttggaaaagt accagcaggg agactttggc tactgtcctc 4654
Tyr Thr Pro *
90

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Met Val

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Lys Leu Tyr Cys Pro Lys Cys Met Asp Val Tyr Thr Pro Lys Ser Ser
95 100 105 110

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aga cac cac cac acg gac ggc gca tac ttc ggc act ggt ttc cct cac 4928
Arg His His His Thr Asp Gly Ala Tyr Phe Gly Thr Gly Phe Pro His
115 120 125

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Met Leu Phe Met Val His Pro Glu Tyr Arg Pro Lys Arg Pro Ala Asn
130 135 140

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cag ttt gta ccc agg tag ggagcaaaga cagtcacaaa ggatcaaaga 5024
Gln Phe Val Pro Arg *
145

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-29-

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Met Val Ser Arg  
150

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155 160 165

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Arg Ala Gln Ser Arg Leu Phe Ala Asp Cys Pro Pro Thr Ser Pro Ser
170 175 180

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gtc tgt gac acc acc att cct ctg ctg cca ccc ttt cag gaa gtc tat 6422
Val Cys Asp Thr Thr Ile Pro Leu Leu Pro Pro Phe Gln Glu Val Tyr
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Gly Phe *
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&lt;223&gt; Antisense Oligonucleotide

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-37-

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&lt;210&gt; 139

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&lt;223&gt; Antisense Oligonucleotide

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&lt;210&gt; 140

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20

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&lt;223&gt; Antisense Oligonucleotide

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